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# Three unusual modifications, a D-arabinosyl, an N-methyl, and a carbamoyl group, are present on the Nod factors of *Azorhizobium caulinodans* strain ORS571

**(nodule-like structures/*Sesbania rostrata*)**

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ABSTRACT **_Azorhizobium caulinodans_ strain ORS571 is a symbiont of the tropical legume _Sesbania rostrata_. Upon _nod_ gene induction with naringenin, strain ORS571 secretes into the culture medium Nod factors that morphologically change the host plant—in particular, deformed root hairs (Hai/Had) and meristematic foci are formed at the basis of lateral roots. The latter infrequently develop further into nodule-like structures. The azorhizobial Nod factors are chitin tetramers or pentamers, N-acylated at the nonreducing-end glucosamine with either vaccenic acid ($C_{18:1}$) or stearic acid ($C_{18:0}$). They, thus, resemble the previously described Nod factors from (brady)rhizobia. The backbone lipooligosaccharide is substituted with unusual modifications, presumably involved in host-specificity determination. There is a D-arabinose branch on the reducing end and an N-methyl and O-carbamoyl substitution on the nonreducing end of the oligosaccharide chain. The previously identified _nod_ gene _nolK_ may be involved in the synthesis of a D-arabinose derivative. The _nodS_ gene product is probably responsible for the N-methylation of Nod factors.**

Bacteria belonging to the genera *Azorhizobium, Bradyrhizobium*, and *Rhizobium* induce the formation of nitrogen-fixing nodules on the roots of specific leguminous host plants. Bacterial nodulation (*nod*) genes, expressed in a host-dependent way, are responsible for the production of molecules, Nod factors, that cause early nodulation events on the host, such as root hair deformation (Had) and meristem formation. The Nod factors made by *Rhizobium meliloti, Rhizobium leguminosarum*, and *Bradyrhizobium japonicum* have a very similar lipooligosaccharide core, with specific modifications (1–3), some of which were correlated with determination of host specificity (2, 4).

*Azorhizobium caulinodans*, a narrow-host-range bacterium related to *Xanthobacter* (5), interacts with *Sesbania rostrata*, a leguminous plant from the Sahel region of West Africa. *S. rostrata* carries vertical rows of dormant root primordia along its stem. These primordia develop into roots when immersed in water; they develop into nodules when infected with *A. caulinodans* (6). *Azorhizobium* also induces nodules at lateral root bases; both stem and root nodules are formed at predetermined sites and develop following a similar pattern after crack–entry infection (7–9).

Previously, we have identified among the azorhizobial nodulation genes two flavonoid-inducible loci, called *nod* loci 1 and 4 and a constitutively expressed regulatory *nodD* gene (10). *nod* locus 1 carries the genes *nodABCSUIJ*, related to corresponding genes from *Rhizobium* or *Bradyrhizobium* (ref. 11; D. Geelen, P.M., R. Geremia, S. Goormachtig, M.V.M., and M.H., unpublished work). Locus 4 carries a gene, *nolK*, with weak similarity to NAD/NADP-requiring sugar epimerases (12). In the present paper, we describe the identification, purification, structure, and biological activity of the azorhizobial Nod factors (NodARc) produced after induced expression of the *nod* genes. These factors consist of a backbone of $\beta$-1,4-linked *N*-acetylglucosamine with an acyl chain replacing the acetyl group on the nonreducing-end sugar. Specific modifications are present, and the putative role of some *nod* gene products in Nod factor decoration will be discussed.

## MATERIALS AND METHODS

**Bacterial Strains and Growth Media.** ORS571 was grown at 37°C on MMO medium [5 g of sodium DL-lactate, 5 g of disodium succinate·6 $H_2O$, 1.67 g of $K_2HPO_4$, 0.87 g of $KH_2PO_4$, 0.1 g of $MgSO_4$·7 $H_2O$, 0.1 g of NaCl, 1 g of $(NH_4)_2SO_4$, 0.04 g of $CaCl_2$·2 $H_2O$, 2 mg of biotin, 4 mg of pantothenic acid, 4 mg of nicotinic acid, and distilled water to a vol of 1000 ml, pH 6.8] (11) supplemented with naringenin when necessary for *nod* gene induction. Other strains used are ORS571-V44 (13) and ORS571(pRG70) (13).

**Detection of Nod Factors by TLC.** Radiolabeling of Nod metabolites was done, as described by Spaink *et al.* (14) with minor modifications. Briefly, an overnight culture was diluted in fresh MMO medium to a $OD_{600}$ of 0.2 unit in 1 ml of final vol; these cultures were preincubated at 37°C for 1 hr before *nod* gene induction with naringenin (10 $\mu$M); 4 hr after induction, [$^{14}$C]acetate (25 $\mu$Ci per ml of culture; 1 Ci = 37 GBq) was added; 12 hr after induction, cells were centrifuged, and the supernatant was extracted twice with 500 $\mu$l of 1-butanol; finally, the butanol extract was dried, redissolved in $H_2O$, and washed with ethyl acetate. NodARc factors were found to be butanol soluble and ethyl acetate insoluble. Extracts were applied to octadecyl silica TLC plates by using $H_2O/CH_3CN$ (1/1) as solvent. Radioactive metabolites were localized by fluorography with 0.4% 2,5-diphenyloxazole in 2-methylnaphthalene (15).

**Purification of Nod Factors.** Cells of overnight cultures, induced with 20 $\mu$M naringenin, were pelleted by centrifugation, and the culture supernatant was filtrated through 0.45-$\mu$m filters. For cultures up to 4 liters, the filtrate was extracted twice with one-fifth vol of 1-butanol; the butanol extract was dried, redissolved in $H_2O$, and washed with 1 vol of ethyl acetate. Alternatively, larger filtrates were extracted with a polystyrene column (XAD-4; Fluka), and products were eluted from the column with $CH_3OH$. The butanol- or polystyrene-extracted products were further purified with $C_{18}$ reverse-phase HPLC (7.5 × 250 mm Spherisorb, Co-

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Abbreviations: FAB, fast atom bombardment; DEPT, distortionless enhancement by polymerization transfer.

lochrom, France, ODS2 5-$\mu$m column) using a gradient from 40% to 80% $CH_3CN$ in 30 min (detection at 206 nm).

**Chemical Analysis of the Nod Factors.** MS was done on a ZAB-HS instrument. In the fast atom bombardment (FAB) ionization mode, a 8-keV xenon atom beam was focused on a target loaded with 1 $\mu$l of sample solution in methanol mixed with 1 $\mu$l of *m*-nitrobenzyl alcohol/glycerol, 1:1 doped with 1% trichloroacetic acid or 1% NaI. NMR spectra were collected on a Varian 600 MHz spectrometer at 25°C with deuterated dimethyl sulfoxide as solvent. Carbohydrate determination and fatty acid analysis were done as described by Roche *et al.* (16). For methylation analysis (17) reduced Nod factors were permethylated by the Hakomori procedure (18). The permethylated oligosaccharide was hydrolyzed with 4 M HCl at 100°C for 8 hr. Partially methylated monosaccharides were reduced with $NaBH_4$ and peracetylated with acetic anhydride/pyridine (1/1). Partially methylated alditol acetates were analyzed by gas chromatography–electron-impact MS. $NaIO_4$ oxidation was done with 500 $\mu$g of product dissolved in 5 ml of 0.1 M acetate, 8 mM $NaIO_4$, pH 5.5, in the dark at 4°C for 100 hr. The reaction was stopped by adding 25 $\mu$l of ethylene glycol and a further incubation of 12 hr. The pH was adjusted to 7, and products were reduced with $NaBD_4$. The reaction products were purified with reverse-phase HPLC as above.

**Biological Tests.** Seeds of *S. rostrata* were surface sterilized and germinated as described (12). Seedlings were transferred to agar slants with Jensen medium (19). Seven days after transfer, roots were inoculated with dilution series of Nod factors or with ORS571.

Microscopic observations were done on undissected roots, fixed in glutaraldehyde (2.75% in 0.1 M sodium cacodylate, pH 7.2) and cleared with sodium hypochlorite (20). Root hairs of cleared roots were visualized by staining with 0.02% methylene blue (20). Observations were with bright- or dark-field microscopy.

## RESULTS

**Detection and Purification of Nod Factors Produced by *A. caulinodans*.** As Nod factors are produced in low amounts by rhizobia, we made use of a ORS571 derivative carrying extra copies of the *nodABCSUIJ* operon on plasmid pRG70. The production of Nod factors by the wild type and ORS571(pRG70) was compared by labeling the molecules *in vivo* with [$^{14}$C]acetate. Products were extracted from the culture supernatant, as described in *Materials and Methods*, and loaded on a TLC plate. On the fluorogram, four spots appeared in the lanes of the naringenin-induced cultures of ORS571 and ORS571(pRG70). These spots were absent in the uninduced cultures or in the cultures of the *nodA* mutant ORS571-V44 (Fig. 1), indicating that these products are *nod* metabolites. In both ORS571 and ORS571(pRG70) supernatants, the same spots were present but with different relative intensity. Radioactivity measurement by liquid scintillation showed that ORS571(pRG70) produces $\approx$5.2 times more Nod factors than the wild type (5200 cpm and 1000 cpm, respectively, for the equivalent of 1-ml cultures supplemented with 25 $\mu$Ci of [$^{14}$C]acetate).

On a large scale, products were obtained by extracting the supernatant of naringenin-induced ORS571(pRG70) cultures with 1-butanol or with polystyrene. The crude extracts were further purified by HPLC (see *Materials and Methods*). The HPLC chromatogram (Fig. 1) shows two major peaks (PI and PII), each accompanied with a smaller peak (PIa and PIIa, respectively). From a 16-liter culture, 27 mg from PI, 23 mg from PII, 3 mg from PIa, and 1 mg from PIIa were obtained. The peaks were absent in the supernatant of an uninduced culture of ORS571(pRG70). Furthermore, these fractions coeluted with the radioactivity when coinjected with radio-

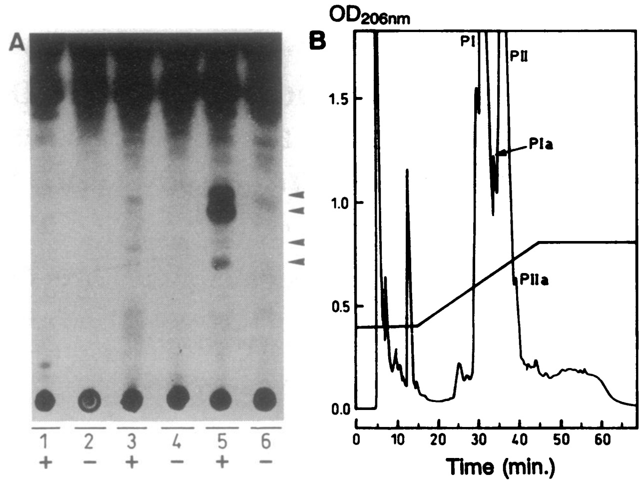



FIG. 1. (*A*) TLC analysis of $^{14}$C-labeled Nod factors produced by ORS571-V44 (*nodA*$^-$ strain) (lanes 1 and 2); ORS571 (wild type) (lanes 3 and 4); ORS571(pRG70) (overproducing strain) (lanes 5 and 6). +, Culture induced with 10 $\mu$M naringenin; −, uninduced culture; Nod metabolites are indicated by arrowheads. Equivalents of 1-ml cultures labeled with 25 $\mu$Ci of [$^{14}$C]acetate were loaded. (*B*) HPLC profile of extract from the culture supernatant of ORS571(pRG70) induced with 20 $\mu$M naringenin. The injection corresponds to the products of a ±800-ml culture. Peaks corresponding to NodARc factors are PI, PIa, PII, and PIIa. The line shows the solvent concentration: initial concentration, 40% $CH_3CN$; final concentration, 80% $CH_3CN$.

active Nod factors, isolated from TLC plates (data not shown). The FAB mass spectra from these products showed that they have a structure related to the Nod factors of *R. meliloti* and *R. leguminosarum* biovar *viciae* (see below). On the basis of these criteria, PI, PIa, PII, and PIIa peaks were identified as NodARc factors.

**Chemical Structure of the Nod Factors.** *FAB–MS.* The fractions PI and PII were analyzed by FAB-MS. The spectra of PI and PII are similar, differing only by two mass units for all peaks—the fragments of PI having the lower mass. As an example, the positive-ion spectrum of PII is shown in Fig. 2. Ionization of PII produced pseudomolecular ions $[M+H]^+$ at $m/z$ 1447, 1404, 1315, and 1272. The couples of ions at $m/z$ 1051 and 1094, $m/z$ 848 and 891, $m/z$ 645 and 688, and $m/z$ 442 and 485 were attributed to the cleavages of glycosidic bonds. The mass difference of either 221 or 203 between fragments corresponds to the dissociation of an *N*-acetylhexosamine-containing oligosaccharide chain. Therefore, the oligosaccharide backbone of PI and PII consists of five hexosamines. Pseudomolecular ions of the couples at $m/z$ 1447 and 1315 and $m/z$ 1404 and 1272 differ by 132 mass units, corresponding to the mass of a pentose substitution, located on the reducing end of the molecule. The 43 mass units difference between these two couples is assigned to a CONH additional group (see below). The variations in the relative abundance of the ions with 43 mass unit difference are sample dependent, suggesting two molecular species and not only molecular and fragment ions. The same is true for the pentose substitution. This result was confirmed by a FAB-MS spectrum in a matrix doped with a sodium salt, where fragmentations are strongly decreased.

The FAB-MS spectra of the minor products PIa and PIIa are, like the spectra of PI and PII, similar with pseudomolecular ions $[M+H]^+$ at $m/z$ 1110 and $m/z$ 1112, respectively, and fragment ions at $m/z$ 889, 686, and 483 for PIa and at $m/z$ 891, 688, and 485 for PIIa. These spectra correspond to molecules consisting of four hexosamines without a pentose substitution, and all have a CONH group on the nonreducing end, as the ions are not accompanied with an ion of 43 mass units less.

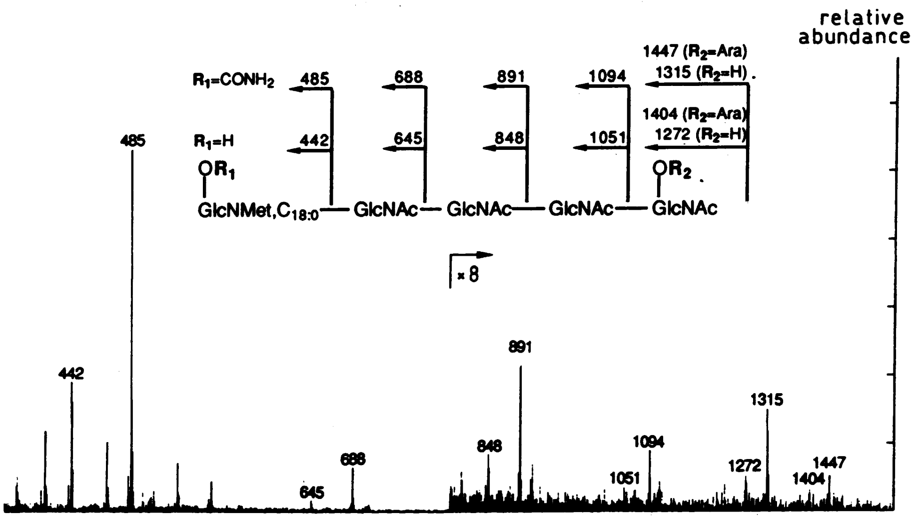



FIG. 2. FAB-MS of peak PII. Fragmentations were assigned as shown above the spectrum. The spectrum shows that PII is a mixture of molecules with and without a pentose substitution and with and without a carbamoyl substitution. The common backbone to all these components consists of a pentamer of *N*-acetylglucosamine, stearic acid, and an N-methyl group. GlcNAc is *N*-acetylglucosamine; GlcNMet,$C_{18:0}$ is glucosamine N-methylated and N-acylated with stearic acid.

*Carbohydrate analysis.* The monosaccharide components of PI and PII peaks were identified by gas chromatography and gas chromatography coupled to MS after methanolysis and peracetylation or pertrimethylsilylation of the methyl glycosides. The chromatogram showed the presence of glucosamine, *N*-methylglucosamine, and arabinose. Glucosamine, *N*-methylglucosamine, and arabinose were assigned to the D series by gas chromatography analysis of their (−)-2-butylglycoside derivatives. D-Arabinose is a pentose corresponding to the substitution of 132 mass units in the molecules with $M_r$ = 1446 and $M_r$ = 1403. The relative intensity of the arabinose peak in the gas chromatogram confirmed that PI and PII are mixtures, containing molecules with and without an arabinose substitution.

*Lipid analysis.* The fragment ions at *m/z* 440 for PI and at *m/z* 442 for PII were assigned to the oxonium ions of the nonreducing-end glucosamine residues. The mass of these fragments and the structure of the Nod factors of (brady)rhizobia suggest that this glucosamine is N-acylated. To identify the acyl groups of PI and PII, the fatty acids were released by alkaline hydrolysis. By gas chromatography analysis of their methyl esters, it was shown that the fatty acids of PI and PII are $C_{18:1}$ and $C_{18:0}$ (stearic acid), respectively. $C_{18:1}$ is vaccenic acid because the double bond is located on position 11, as deduced from the collision-activated dissociation mass analyzed ion kinetic energy (CAD-MIKE) spectrum of its carboxylate anion (data not shown). The presence of one double bond in the fatty acid of PI and no double bond in the fatty acid of PII explains the more hydrophobic nature of PII and the difference of two mass units for all peaks in the respective FAB-MS spectra.

*Substitutions of the nonreducing-end glucosamine.* The calculated mass of an oxonium ion of glucosamine, substituted with $C_{18:1}$ or $C_{18:0}$, is 426 or 428, respectively; this is a difference of 14 mass units with the actual masses as detected in the mass spectra of PI and PII. Fourteen mass units corresponds to a methyl substitution, which agrees with the observed *N*-methylglucosamine as a constituent of PI and PII. The N-methyl carbon appeared at 30.7 ppm in the $^{13}C$ distortionless enhancement by polymerization transfer (DEPT) NMR spectrum (Fig. 3*D*); the proton resonance of this group is at 2.9 ppm in the $^{1}H$ NMR spectrum (data not shown).

The fragment ions at *m/z* 483 for PI and *m/z* 485 for PII correspond to the same nonreducing-end glucosamine but substituted with the group of 43 mass units, which is identified as a carbamoyl group by $^{13}C$ NMR: the resonances at 159.6 ppm (Fig. 3*A*) correspond to the value reported for a

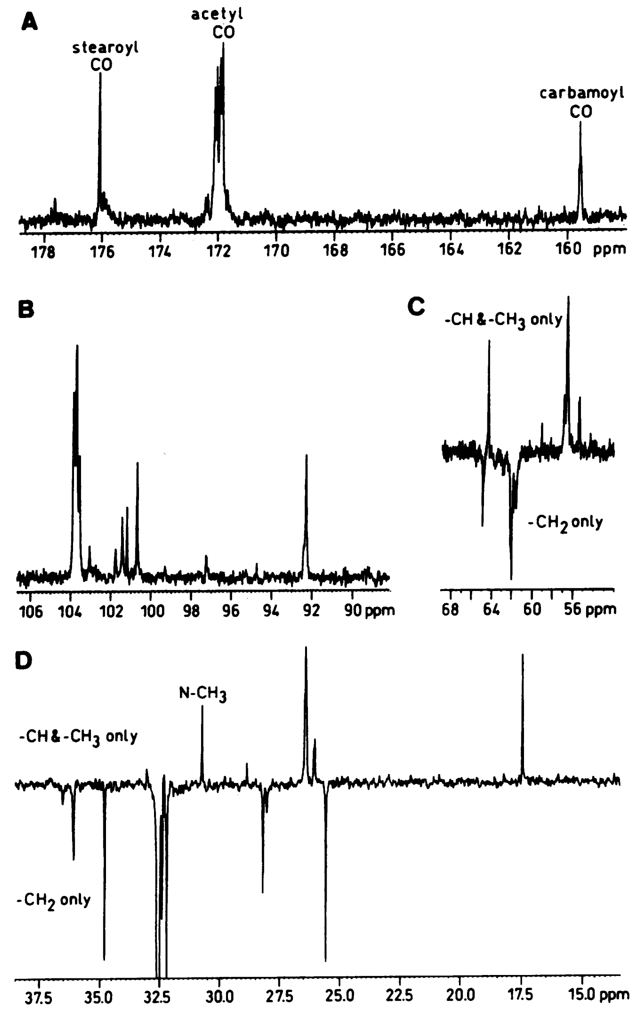



FIG. 3. $^{13}C$ NMR spectrum of PII. (*A*) Carbonyl region. (*B*) Anomeric region. (*C*) C6 of the *N*-acetylglucosamine residues (DEPT). (*D*) Aliphatic region (DEPT).

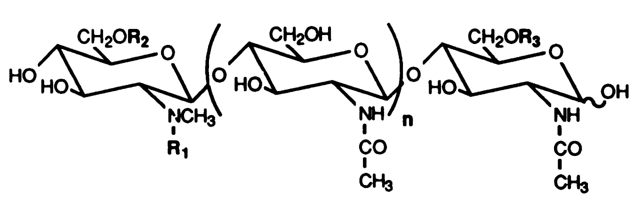
carbamoyl carbonyl carbon (21). In a $^{13}C$ NMR DEPT experiment, a down-field shift of 2.9 ppm was observed for a C6 of glucosamine (Fig. 3*C*), showing that the carbamoyl group is positioned on C6 (22). The nonreducing-end glucosamine substituted with the carbamoyl group can be oxidized upon treatment with $NaIO_4$, again indicating that the carbamoyl group is on C6.

*Glycosidic linkages.* By methylation analysis of PI and PII reduced with $NaBD_4$, we found 1,4 linkages between the glucosamine residues. The $^1H$ and $^{13}C$ NMR spectra showed a $\beta$-anomeric configuration for the glucosamines except for the reducing end present in both the $\alpha$ and $\beta$ forms (Fig. 3*B*). Thus, the linkages between the glucosamines are $\beta$-1,4, as observed in chitin and Nod factors of (brady)rhizobia.

The methylation analysis showed a reducing-end *N*-acetylglucosamine that is not methylated on C4 and C6, indicating that the arabinose is linked on C6. Also a reducing-end *N*-acetylglucosamine, linked only on C4, was detected, again indicating that PI and PII are mixtures containing molecules with and without an arabinose substitution. The anomeric configuration of the D-arabinose is $\beta$, as the $^{13}C$ NMR spectrum shows a resonance at 101.4 ppm (Fig. 3*B*), typical for $\beta$-D-arabinoside (23).

Fig. 4 shows the structure of the ORS571 Nod factors deduced from the above data. In conclusion, the NodARc factors are chitin tetramers or pentamers N-acylated at the nonreducing end with either vaccenic or stearic acid. Part of the pentamers are branched at the reducing end with D-arabinose. The nonreducing end is substituted with an N-methyl group in all molecules and a carbamoyl group on part of the pentamers and all of the tetramers.

**Biological Activity of PI and PII.** The purified Nod factors of ORS571 were tested for biological activity on the roots of the host plant *S. rostrata*. Because methods for the separation of the components of PI and PII have yet to be developed, the mixtures PI and PII were tested. Four days after inoculation, PI and PII elicited the formation of new root hairs, very localized, at the basis of the secondary roots (Hai). These newly formed root hairs are deformed, showing curls or branches (Had) (Fig. 5). Uninoculated plants or plants inoculated with ORS571 have no root hairs at these sites. Six days after inoculation with Nod factors, small swellings due to cell divisions were visible at the basis of the secondary roots (Fig. 5). Most of these swellings did not develop further. However, with a very low frequency, they did develop into nodule-like structures (Fig. 5). These nodule-like structures were distinguishable from real nodules by the absence of the characteristic pink color that is due to the presence of leghemoglobin in fixing nodules. The minimal concentration of PI or PII needed to detect Hai was $10^{-9}$ M. This amount is significantly higher then the minimal concentration ($10^{-11}$ M–$10^{-12}$ M) needed for Had activity by the Nod factors of *R. meliloti*, *R. leguminosarum*, and *B. japonicum* (1–3). Perhaps Had and Hai are different responses that need different activation mechanisms. Another possibility is that the mixtures PI and PII produced by the strain ORS571-(pRG70) contain molecules that have no activity or even inhibitory effects. No difference in activity between PI and PII was detected.



$R_1$ = stearoyl ($C_{18:0}$) or vaccenoyl ($C_{18:1}$)
$R_2$ = carbamoyl ($CONH_2$) or H
$R_3$ = D-arabinosyl or H
n = 2 or 3

FIG. 4. Chemical structure of the NodARc factors. Nomenclature rules for Nod factors were proposed by Roche *et al.* (4). According to these rules a NodARc molecule that is a pentamer of *N*-acetylglucosamine, substituted with D-arabinose, a carbamoyl group, an N-methyl group, and vaccenic acid ($C_{18:1}$), is named NodARc-V(Ara,Carb,Met)(18:1).

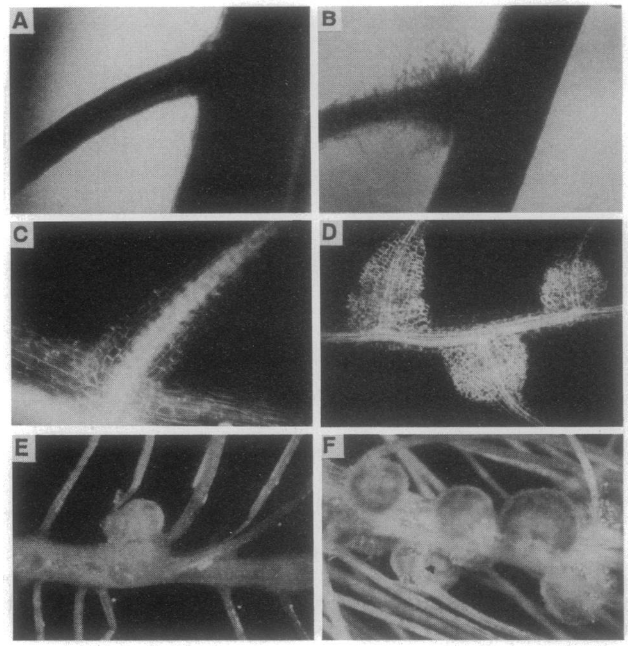



FIG. 5. Effects of purified NodARc factors on the roots of *S. rostrata*. (*A* and *C*) Uninoculated roots. (*B*) Root hair induction and deformation by PI ($10^{-9}$ M). (*D*) Swellings induced by PI ($10^{-6}$ M). (*E*) Nodule-like structure induced by PII ($10^{-7}$ M). (*F*) Nodules formed by strain ORS571. (*A* and *B*, ×12.5; *C*, ×25; *D*, ×6; *E* and *F*, ×3.)

## DISCUSSION

Radiolabeling with [$^{14}C$]acetate and extraction with 1-butanol allowed the identification of Nod factors in the supernatant of naringenin-induced cultures of *A. caulinodans*. For purification, an overproducing strain was used with extra copies of the *nodABCSUIJ* operon. This strain secretes a population of Nod factor molecules that were separated by HPLC in four fractions (PI, PIa, PII, and PIIa) (Fig. 1). The structure of these products was determined by using mass and NMR spectrometry, gas chromatography, and methylation analysis. The backbone of the molecules consists of a tetramer (for PIa and PIIa) or pentamer (for PI and PII) of $\beta$-1,4-linked glucosamine residues. The amine at the nonreducing end is substituted with either vaccenic acid ($C_{18:1}$) for PI and PIa or stearic acid ($C_{18:0}$) for PII and PIIa, whereas the other glucosamines are N-acetylated. Vaccenic acid and stearic acid are common acyl chains to Gram-negative bacteria. Vaccenic acid was also detected in the Nod factors of *B. japonicum* (3) and in part of the *R. leguminosarum* Nod factors (2). In contrast, the *R. meliloti* factors and the other fraction of the *R. leguminosarum* factors carry *nod*-specific fatty acids with one to three double bonds conjugated with the carbonyl of the amide (1, 2, 24). A number of unusual modifications are present on the lipooligosaccharide backbone of the azorhizobial Nod factors. In ± 30% of the molecules the reducing-end glucosamine is branched on C6 with the pentose sugar D-arabinose. The glucosamine at the nonreducing end has two substitutions: the amine is always methylated, and in ≈60% of the molecules, a carbamoyl group is present on C6. It is tempting to speculate about an analogous role in Nod factor function for the N-methyl group in NodARc and the conjugated double bonds in the Nod factors of *R. meliloti* and *R. leguminosarum*. The D-arabinose, N-methyl, and carbamoyl modifications of NodARc are possibly involved in the host specificity determination of ORS571. Further studies are necessary to confirm this hypothesis.

Inoculation of the roots of *S. rostrata* with the mixtures PI or PII in concentrations of $10^{-6}$–$10^{-9}$ M resulted in the formation of deformed root hairs and meristematic foci at lateral root bases that occasionally develop further into nodule-like structures. The latter phenomenon occurred only in the presence of high concentrations of Nod factors ($10^{-6}$ M–$10^{-7}$ M). The formation of nodule-like structures by pure Nod factors has been described for the *R. meliloti*/alfalfa symbiosis (25) but not for any other interaction. The finding that azorhizobial Nod factors induce the formation of root hairs on *S. rostrata* was unexpected, as root hairs are not involved in the infection pathway on *S. rostrata*. However, it has recently been shown that infection thread and root hair formation may initially share a common morphogenetic pathway that ultimately leads to root hair formation when Nod factors alone are applied and to infection thread formation in the presence of bacteria (26).

D-Arabinose is a rare sugar and, probably, the bacterium has to make an activated form of it for factor synthesis. The gene *nolK* is a candidate for such a function: it has a NAD/NADP-binding site and is homologous to sugar epimerases (12). We found that the protein encoded by the gene *nodS* contains a motif that is conserved among methyltransferases that use *S*-adenosylmethionine as cofactor (D. Geelen, P.M., R. Geremia, S. Goormachtig, M.V.M., and M.H., unpublished work). There is strong evidence that this gene is responsible for the N-methylation of the azorhizobial Nod factors. Taking into account the complexity of the ORS571 Nod factors and the number of characterized *nod* genes in this strain, it is likely that ORS571 has still unidentified *nod* genes—e.g., arabinosyltransferase and carbamoyltransferase genes.

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